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Drosophila As a Model System for Molecular Analysis of Tumorigenesis

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In Drosophila, homozygous mutations in a series of genes can cause the appearance of tissue-specific tumors. These tumors occur either during embryonic or larval development. The majority of the identified genes give rise to larval tumors that affect either the presumptive adult optic centers of the brain, the imaginal discs, the hematopoietic organs, or the germ cells. These genes act as recessive determinants of neoplasia and have been designated as tumor-suppressor genes. They are normally required for the regulation of cell proliferation and cell differentiation during development. Among these genes, the lethal(2)giant larvae (*l(2)gl*) has been best studied. Homozygous mutations in *l(2)gl* produce malignant tumors in the brain hemispheres and the imaginal discs. The *l(2)gl* gene has been cloned, introduced back into the genome of *l(2)gl*-deficient animals, and shown to restore normal development. The nucleotide sequence of the *l(2)gl* gene has been determined, as well as the sequence of its transcripts. Anti-*l(2)gl* antibodies recognize a protein of about 130 kDa that corresponds to the major product of l(2)gl transcripts. Analysis of the spatial distribution of l(2)gl transcripts and proteins revealed a first phase of intensive expression during embryogenesis and a second weaker phase during the larval to pupal transition period. As revealed by mosaic experiments, the critical period of *l(2)gl* expression for preventing tumorigenesis takes place during early embryogenesis. During this period, the l(2)gl protein is ubiquitously expressed in all cells and tissues, while during late embryogenesis expression becomes gradually restricted to the midgut epithelium and the axon projections of the ventral nervous system that show no phenotypic alteration in the mutant animals. Biochemical studies revealed that the l(2)gl protein can be metabolically labeled with phosphate and is associated with the cytoplasmic face of the plasma membrane. Sequence comparison has further shown that the l(2)gl proteins contain reiterated sequence motifs that are conserved in G proteins. These data present compelling evidence to implicate *l(2)gl* in a signal transduction process that ultimately controls cell proliferation and differentiation.

Introduction

Genetic and molecular analyses of Drosophila have clearly demonstrated that tumorigenesis may arise from inactivation of genes controlling cell growth and differentiation (*1,2*). Recessive mutations in a series of genes were shown to interrupt the differentation of defined adult primordial cell types and lead to an uncontrolled and invasive cell growth, resulting in the death of the mutant animals before the completion of the normal course of development.

In Drosophila, malignancies of genetic origin occur either in the brain, the imaginal disc cells, or the hematopoietic cells (*3–5*). With the exception of the lethal(2)giant larvae (*l(2)gl*) gene, whose inactivation results in the appearance of tumors in two types of tissues, brain and imaginal discs (*1,6*), all other mutations produce neoplasia in only one type of tissue (*3–5*). In general, all the animals homozygously mutated at one gene locus display a similar pattern of neoplasia. For example, in the case of *l(2)gl*, every single imaginal disc becomes tumorous, and no variation can be observed from animal to animal. This reproducible pattern of tumors indicates that no secondary genetic event is required for eliciting full malignant transformation.

This is in contrast to tumor development in mammals and particularly in humans, where this process appears generally to be complex and results from accumulation of several genetic and epigenetic changes (*7–9*). Recent studies on small cell lung cancer, breast cancer, and colorectal cancer have shown that the full malignant condition is associated with multiple genetic changes, supporting the concept of a multigenetic etiology of cancer (*11–14*). However, certain childhood malignancies,

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such as retinoblastoma, seem to originate from genetic alterations of only a single gene (*15–17*).

Isolation of the putative retinoblastoma (*Rb*) gene has confirmed that inactivation of *Rb* constitutes the primordial event in the malignant transformation of human retinal cells (*18–21*). In addition, inactivation of the *Rb* gene has also been found to be common in other human malignancies such as osteosarcomas, small-cell lung carcinomas, breast carcinomas, bladder carcinomas, and cervical carcinomas (*19,22,23*). This indicates that the *Rb* gene also plays a critical role in the control of neoplastic growth in these tissues, albeit not as cardinal as in the case of retinal cells. Genetic and molecular studies suggest strongly that further genetic alterations may be required for the full transformation of these tissues. The main candidates for involvement in these genetic events are proto-oncogenes and tumor-suppressor genes. Modifications in the structure and/or control of these genes could induce the malignant phenotype.

Over the past decade, a series of dominantly acting oncogenes has been detected through its ability to induce cell proliferation and tumorigenesis. The oncogenes are derived from deregulation or structural alterations of the products of proto-oncogenes that are components of the normal cellular genome. These genes encode growth factors, growth factor receptors, signal transducers, protein kinases, and transcription factors whose activities are increased in their overexpressed or mutated forms.

However, earlier genetic analysis of inherited childhood malignancies (*15*) and familial tumor syndromes (*24*), as well as studies with somatic cells (*25–27*), have revealed the existence of a countervailing class of genetic elements whose normal activity is to control cell proliferation. These genes have been designated recessive oncogenes, anti-oncogenes, or tumor-suppressor genes. Inactivation of these genes by deletion or point mutations disrupts the checks and balances that regulate cell proliferation and development.

In Drosophila, both classes of cancer genes, tumor-suppressor genes and proto-oncogenes, have been identified. On one hand, tumor-suppressor genes were first found by virtue of loss of genetic function that results in the appearance of malignancies in the homozygously mutated animals (*1,3–6*). This finding led to the hypothesis that a class of genetic elements exists that must be inactivated by deletion, point mutation, or translocation before an animal can develop malignancies. Final proof of this hypothesis was obtained when the *l(2)gl* gene was cloned, introduced back into the genome of *l(2)gl*-deficient animals, and shown to restore normal development (*2,28,29*).

On the the other hand, proto-oncogenes were detected in the genome of Drosophila by molecular hybridization at low stringencies with vertebrate oncogenes. Other proto-oncogenes have been also identified by finding Drosophila genes displaying similarity between the amino acid sequence of a cloned Drosophila gene and the amino acid sequence of a vertebrate oncogene [for review, see Shilo (*30*) and Hoffman (*31*)]. While numerous Drosophila proto-oncogenes have been identified, mutant alleles are currently available for only a subset of them. Although the studies of these mutations have revealed that the Drosophila homologues to vertebrate proto-oncogenes participate in essential processes throughout the development, none of these mutations was found to induce malignancies or developmental abnormalities that would phenotypically resemble one of those that characterize tumor progression in higher vertebrates, i.e., preneoplastic lesions like hyperplasia.

However, hyperplasia resulting from genetic alterations has also been recognized in Drosophila. Characterization of a series of recessive lethal mutations has revealed that five mutations, designated as epithelial overgrowth mutants, can give rise to overproliferation of the imaginal discs (*33,34*). Although these discs grow to several times their normal size, they maintain a single-layered epithelial organization and retain their capacity to differentiate.

In this report, we briefly review the current state of information regarding tumorigenesis in Drosophila and then focus on our current view of one of the members of the tumor-suppressor genes thus far identified in invertebrates: the lethal(2)giant larvae gene in Drosophila.

Drosophila Neoplasia

The possibility that mutations can cause the formation of tumors was envisaged by Drosophila investigators in the early period of genetic endeavor at the beginning of this century. The first mutation giving rise to a so-called tumor (*35*) was found in the strain *l(1)17* by Bridges in 1916; a lethal allele of deep-orange, *dor* (*36*). However, the death of the $dor^{l(1)17}$ larvae was later found to be caused by a midgut malformation (*37*). These larvae form melanotic masses that morphologically resemble tumors but are devoid of truly neoplastic growth (i.e., the hemocytes aggregate in melanizing nodules and are unable to proliferate further). Upon the failure of 50 years of genetic analysis to obtain convincing data showing that insects can suffer from cancer, it was generally considered that insects were not able to produce any neoplasm comparable to those occurring in vertebrates (*38*). The occurrence of true neoplasms in *Drosophila melanogaster* was only recognized after the isolation and characterization of a new allele of the *l(2)gl* gene by Gateff and Schneiderman (*1*). Developmental studies of *l(2)gl* animals revealed that one of the manifestations of the mutation was the formation of malignant neuroblastoma and the appearance of imaginal disc tumors. Subsequently, other tumor mutations were identified and shown to affect distinct types of tissues: embryonic tissues, the optic centers of the larval brain, the imaginal discs, the hematopoietic organs, and the germ line cells. Altogether, 27 tumor genes have been reported in the literature. Mutants in 17 of them currently exist in published stock lists (*5*) and are listed in Table 1.

Table 1. Neoplastic and hyperplastic mutants in *Drosophila melanogaster*.

Mutant	Symbol	Locus	Map position
A. Neoplastic mutants			
A. 1. Mutants producing embryonic neoplasms[a]			
Df(1)Notch		1-3.0	3B4-E1
shibire^{ts1}	*shi^{ts}*	1-52.2	
A.2. Mutants producing larval neoplasms[b]			
A.2.1 Brain and imaginal disc neoplasms			
lethal(2)giant larvae	*l(2)gl*	2-0.0	21A
A.2.2. Brain neoplasms			
lethal(2)37Cf	*l(2)37Cf*	2-53.9	37C5-7
lethal(3)malignant brain tumor	*l(3)mbt*	3-93	97F
A.2.3. Imaginal disc neoplasms			
lethal(1)discs-large 1	*l(1)d.lg-1*	1-34.8	10B8-9
lethal(2)tumorous discs	*l(2)tud*	2-104	59F5-8
A.2.4 Blood cell neoplasms			
lethal(1)malignant blood neoplasm	*l(1)mbn*	1-27.6	8D
Tumorous lethal	*Tum*	1-34.5	
lethal(3)malignant blood neoplasm-1	*l(3)mbn-1*	3-13.3	64F3-65A3
lethal(3)malignant blood neoplasm-2	*l(3)mbn-2*	3.	
A.3. Germ line neoplasms[c]			
female sterile(1)231	*fs(1)231*	1-	
female sterile(1)1621	*fs(1)1621*	1-11.7	
fused	*fu*	1-59.5	
female sterile(2) of Bridges narrow	*fs(2)B*	2-5	
benign(2)gonial cell neoplasm	*nw*	2-83	
B. Hyperplastic mutants[d]	*b(2)cgn*	2-106.7	60A3-7
lethal(2)fat	*l(2)ft*	2-12	24D8
lethal(2)giant discs	*l(2)gd*	2-42.7	32AE
lethal(3)c43	*l(3)c43*	3-49.0	85E
lethal(3)disc overgrowth-2	*l(3)dco-2*	3-70.7	
lethal(3)disc overgrowth-1	*l(3)dco-1*	3-	100A1.2-B1

[a]The mutants develop abnormalities during embryonic development and die during embryogenesis. Neoplastic potential of the embryonic tissues is assayed by transplanting them into the abdomen of adult flies in which the neoplastic cells proliferate rapidly, form tumors, and kill the host.

[b]The mutants develop neoplasia during larval development and die as third-instar larvae, pseudopupae, or pupae.

[c]The mutants develop benign tumors that remain confined to the gonads and that lead only to sterility without otherwise impairing the development of the mutant animals.

[d]The mutants develop overgrown imaginal discs during larval development and die as third-instar larvae, pseudopupae, or pupae.

Growth Potentialities during Drosophila Development

Before committing to neoplastic growth, a cell must be able to duplicate all its essential parts. This ability is normally limited to undifferentiated cells that are either in a proliferative phase or quiescient but readily recruitable into mitosis. During Drosophila development, two major proliferative phases take place. First, a relatively short but intense phase of cell proliferation occurs during the first 3 hr of Drosophila embryogenesis and, after 16 rounds of mitosis, leads to the formation of two classes of embryonic cells: those that will form the larval tissues and will grow mainly by expansion of cell volume with continued rounds of DNA replication without cytokinesis and resulting in chromosome polytenization, and those that will constitute the anlagen of the adult organs. These latter cells remain quiescent and undifferentiated during the remaining period of embryonic development. During larval life, they resume proliferation from the middle of the first larval instar and divide throughout the three larval stages up to the larval-pupal transition, arresting only as terminal differentiation occurs.

Therefore, cell overproliferation can only be noticed at the end of both major proliferative phases, and, accordingly, mutations producing neoplasia have been identified at the end of either one or the other period.

Phenotypic Characteristics of Drosophila Neoplasia

Embryonic neoplasia does not form visible tumors. Therefore, an assay is required for recognizing neopla-

sia in embryos. For this purpose, fragments of putative neoplastic tissues are implanted into the abdomen of adult flies. Under these conditions, the neoplastic cells proliferate rapidly, invade the abdominal cavity, and kill the host in 3 to 14 days (*1,6,34*). By contrast, normal tissues grow moderately and exert no deleterious effect on the host. This procedure provides an efficient system for distinguishing between normal and neoplastic tissues. Through this assay, two mutations giving rise to embryonic neoplasia, *Df(1)Notch* and *shibire*, have been identified (*6*).

Larval neoplasia produces massive outgrowths of tumorous tissues that become clearly visible during the several-day extension of larval life. During this period, the tumorous tissues may reach several times their normal size, and the mutant larvae become either bloated (or "giant") and transparent when they have brain or imaginal disc tumors or white opaque or completely melanized when they are affected by blood neoplasia. In the transplantation assay, brain, imaginal disc, and blood neoplasia proliferate rapidly and, in few days, lead to the death of the host (*34*).

Cell overproliferation affecting the germ line is essentially detected in the adult organism in which it causes sterility. Overgrowth remains confined within the gonads and does not otherwise impair the development of the fly. Similarly, when transplanted into an adult host, these cells remain confined within the transplanted gonads and are unable to sustain an autonomous growth. Mutations giving rise to germ line overproliferation affect essentially the female germ line, with the exception of the *b(2)gcn* mutation that gives rise to overproliferation of both male and female germ line (*39*). These mutations are currently considered as affecting the sex determination of the germ line rather than causing true neoplasms.

Similarly, overgrowth of the imaginal discs produced by the epithelial overgrowth mutations does not show characteristics of malignant neoplasms. These overgrown discs maintain a single-layered epithelial structure, with normal apical-basal cell polarity, and conserve their ability to differentiate, albeit forming abnormal cuticular structures.

In general, Drosophila neoplasms display a range of characteristics similar to those defining neoplasms of vertebrates. The main characteristics are the following: *a) in situ* cell overproliferation, *b)* altered cell morphology, *c)* loss of differentiation capacity, *d) in situ* invasiveness, and *e)* transplantability. However, these features characterize essentially the most malignant tumors, and, as in vertebrate neoplasms, Drosophila tumors may display only some of these properties. In particular, the *l(3)mbn* hemocytes proliferate and invade all larval tissues but do not proliferate in adult hosts (*40*).

Finally, in addition to the appearance of malignant neoplasms, these mutations may exert deleterious effects on other tissues. In particular, the *l(2)gl* mutation affects the development of numerous tissues (i.e., atrophy of the male germ line, underdevelopment of the imaginal cells of the salivary glands and the gut) before the appearance of the overgrown neoplasms (*41,42*). Therefore, these mutations produce pleiotropic effects, neoplasia being the most striking feature.

Genetic analysis of Drosophila strongly supports the concept that cell proliferation and cell differentiation may be negatively regulated. The importance of the genes involved in these processes has quite recently come to the forefront. The techniques of molecular biology used for the analysis of oncogenes have already greatly increased our understanding of the processes required to control normal and defective proliferation. However, genetic analysis of proto-oncogenes has clearly shown that, in Drosophila, these genes cannot be causally related with tumor development. From the molecular isolation of tumor-suppressor genes and their analysis, we can expect numerous exciting discoveries. These findings will help to dissect and identify significant regulatory components of cell proliferation and may offer great hope for further insights into the basic mechanisms leading to tumorigenesis.

The *l(2)gl* Gene

The lethal(2)giant larvae gene was the first molecularly isolated tumor-suppressor gene (*2*). Furthermore, integration of a normal copy of this gene into the genome of *l(2)gl*-deficient animals was shown to restore normal development and consequently prevent tumorigenicity.

Homozygous mutations in the *l(2)gl* gene lead to malignant transformation of the neuroblasts and ganglion mother cells of the adult optic centers in larval brain and of the imaginal disc cells (*1,6*). Among Drosophila cancer genes, the mutations in the *l(2)gl* gene are the only mutations giving rise to simultaneous appearance of malignancies in two distinct tissues. In homozygous *l(2)gl*-deficient animals, these neoplasms first become visible in the third larval instar. The brain hemispheres and the imaginal discs grow to several times their normal size during the extended life of the *l(2)gl* larvae. Presumably, the invasive growth of the malignant neuroblasts within the brain hemispheres disrupts essential functions for the maturation of the ring gland and its ecdysone-producing cells (*43,44*). As a consequence, the ring gland remains underdeveloped and, because of the lack of ecdysone, puparation is considerably delayed. However, the atrophy of the ring gland is apparently not the primary defect in the mutant animals because neither the transplantation of a normal ring gland into *l(2)gl*-deficient larvae (*45*) nor the injection of ecdysone (*46*) fully rescue the development of the mutant animals. Furthermore, ecdysone alone is not sufficient to cause neoplastic growth (*47,48*). Other tissues like the fat bodies become involuted, but their atrophy seems to be caused by the development arrest resulting from the occurrence of malignancies and may not be related to the absence of *l(2)gl* gene expression in these tissues. By contrast, the underdevelopment of tissues, such as the gonads and the imaginal cells of salivary glands and the gut, occurs before the outgrowth of the tumors and

can be directly attributed to the absence of *l(2)gl* gene activity in these tissues (*42*). For example, in *l(2)gl*-deficient animals, the development of the male germ line is arrested during early larval life, whereas the development of the female germ line proceeds further (*49*).

The most striking characteristic of the *l(2)gl* phenotype is certainly the neoplastic growth affecting the optic centers of the larval brain and the imaginal discs. When fragments of both of the overgrown tissues are transplanted into the abdomen of adult wild-type flies, the tissues behave as malignant tumors. They grow autonomously and kill the host fly within 1 to 2 weeks (*1,6*).

The first *l(2)gl* mutant was discovered by Bridges in 1933 (*50*) and has been mapped to position 21A-B at the extreme left end of the second chromosome (*51*).

Molecular Identification of the *l(2)gl* Gene

In situ hybridization to polytene chromosomes of genomic DNA sequences expressing pole-cell-specific transcripts revealed that one of the isolated sequences derived from the 21A region (*2*) where the *l(2)gl* was previously located (*51*). Assignment of this DNA segment to the *l(2)gl* locus was further reinforced by analyzing polytene chromosomes heterozygous for *l(2gl* mutations. This analysis showed that the cloned DNA segment was deleted in $l(2)gl^{1}$ and $l(2)gl^{4}$, two independently isolated *l(2)gl* mutant alleles (*2*). Subsequent analysis of more than 50 spontaneously occuring and ethyl methane sulfonate-induced *l(2)gl* mutations by Southern genomic blotting confirmed that almost all *l(2)gl* mutations consisted of relatively large deletions in which part or all of the DNA sequence between the telomere and the cloned region was absent. However, two mutants, $l(2)gl^{GB52}$ and $l(2)gl^{DV275}$, displayed structural alterations within the cloned region. The $l(2)gl^{DV275}$ mutation exhibits an interstitial deletion of about 9 kb, whereas the $l(2)gl^{GB52}$ is characterized by the insertion of a 10 kb DNA which was later identified as a mobile element of the B104 family (*52*). Finally, Northern blot analysis has led to the delimitation of a transcription unit which is altered in all examined *l(2)gl* mutant alleles. The putative l(2)gl transcription unit encompasses a 13.1 kb segment of DNA that in subsequent studies was shown to contain all the necessary sequences for correct expression.

l(2)gl Gene Transfer and Gene Rescue

In Drosophila, functional activity of a cloned gene can be tested by generating transgenic flies through introduction by P-element-mediated gene transfer (*53,54*). In the case of *l(2)gl*, the 13.1 kb EcoRI DNA fragment covering the putative l(2)gl transcription unit was incorporated into a P-element transposon (*28*). This construct was microinjected into embryos of Drosophila heterozygous for *l(2)gl*. Transgenic animals were selected and backcrossed so that isogenic lines could be obtained. From the segregation of the progeny, it was clear that the cloned sequence was able to fully rescue the development of *l(2)gl*-deficient animals that otherwise would have died of brain and imaginal disc tumors. These results demonstrate that all the necessary sequences for *in vivo* function of the *l(2)gl* gene are included within the 13.1 kb DNA fragment and show that the development of brain and imaginal disc tumors results from the absence of *l(2)gl* function (*28*). When this function is restored, tumorigenesis is completely suppressed.

Structure of the *l(2)gl* Gene and Its Transcripts

The entire 13.1 kb DNA segment encoding the *l(2)gl* sequence, as well as several cDNAs, has been sequenced (*29*). This analysis was revealed that the *l(2)gl* gene can be divided in roughly two equal moieties. The proximal moiety is made of two direct repeats, each about 2.8 kb in size. These repeats show a high degree (96%) of homology. Sequence analysis of a series of cDNAs, S1 mapping, and primer extension experiments have shown that initiation of transcription can occur in both repeats, at about 0.4 kb upstream from their proximal limits (*55*; M. Schmidt, I. Török, and B. Mechler, in preparation). Depending on the splicing pattern, this duplication leads to the synthesis of transcripts that differ in their 5′ region. However, these variations affect only the 5′ untranslated region and therefore bear no consequence on the l(2)gl protein structure. Moreover, as shown by gene transfer experiments, the promoter present in each repeat can fully direct the *l(2)gl* gene expression. Further analysis of homologous sequences to *l(2)gl* in related Drosophila species, such as *D. simulans*, *D. mauritiana*, *D. erecta*, and *D. yakuba*, indicated that the duplication of the proximal moiety of the gene is likely to be a relatively recent event (between 1 and 2 million years ago) since it is only found in *D. melanogaster* and is absent in all other investigated Drosophila species (P. Mendes and B. Mechler, unpublished observation).

The distal moiety of the *l(2)gl* gene contains almost all the coding sequence of the gene. Sequence alignment of the longest isolated cDNA, *Ec173*, revealed that this cDNA extends over 10 exons (*29*). The first three exons derive from the duplicated 5′ region, whereas the seven other exons containing almost all the coding region of the gene originate from the unique 3′ region. Sequence analysis of this cDNA indicated that the longest open reading frame encodes a protein of 1161 amino acids in length with a relative mass of 126.9 kDa: designated as p127 protein. From sequence divergence found in another cDNA, *Ec371*, a second l(2)gl protein can be predicted with a length of 708 amino acids and a relative mass of 78.1 kDa, designated as p78 protein (*29*).

The divergence found in the coding sequence was assumed to represent the two size classes of l(2)gl transcripts that were previously observed in Northern blot analysis (*2*). However, results from several lines of in-

vestigation, such as structural analysis of the transcripts, gene dissection and reconstitution studied in transgenic flies, and protein analysis, suggested that the 4.5-kb transcripts may correspond to cleaved products of the 6 kb transcripts. This cleavage may represent a degradation process affecting the 3′ untranslated portion of the l(2)gl transcripts that contain numerous AUUUA repeats to confer mRNA lability (*56*; M. Schmidt, I. Török, and B. Mechler, in preparation).

Structure of the l(2)gl Polypeptide

From structural and sequence analyses of the l(2)gl transcripts, it appears that the *l(2)gl* gene encodes essentially a polypeptide of about 127 kDa in size. using rabbit antisera prepared against a β-galactosidase-l(2)gl fusion protein, a major protein of an apparent molecular mass of about 130,000 can be identified on Western blot or can be immunoprecipitated.

Sequence comparison of the putative l(2)gl protein with protein sequences in the general data bases has not shown any significant homology with any of the proteins compiled. Examination of the sequence did not reveal any obvious pattern that would indicate a specific function (e.g., zinc finger, leucine zipper, or other) or a specific subcellular localization (e.g., NH_2-terminal hydrophobic signal secretion sequence, potential hydrophobic transmembrane domain, karyophilic domain, or other) of the *l(2)gl* sequence. On the basis of the presence of an Arg-Gly-Asp (RGD) sequence, characterizing cell adhesion molecules and the arrangement of Cys residues, it has been suggested that the l(2)gl protein is homologous to neural cell adhesion molecule (N-CAM) (*57*). However, detailed examination of the l(2)gl protein sequence did not show any relationship to the immunoglobulin supergene family to which N-CAM belongs (*58*). Moreover, we have found that the RGD sequence is absent in the putative homologous protein sequence of other insects such as *Calliphora erythrocephala* (*59*; I. Török and B. Mechler, in preparation). A further report, published by the same group of investigators, consider that the l(2)gl protein may be homologous to a cadherin cell adhesion molecule because the l(2)gl protein appears to be localized at the surface of embryonic cells (*60*). However, this contention is not substantiated when these described similarities are compared with corresponding domains of homologous sequences to *l(2)gl* identified in other insects (I. Török and B. Mechler, in preparation).

Cellular Localization of the l(2)gl Protein

Immunostaining of sections of Drosophila embryos has revealed that the l(2)gl protein is localized at the cell periphery (*55,57*). Because no N-terminal secretion signal sequence or transmembrane domain could be identified in the l(2)gl protein, we have investigated the association of the l(2)gl protein with with the plasma membrane. Using cell fractionation procedures, we were able to show that the l(2)gl protein is tightly associated with membrane and cannot be released by high salt washes or EDTA treatment (D. Strand, A. Kalmes, R. Merz, and B. Mechler, in preparation). The orientation of the l(2)gl protein with regard to plasma membrane was further studied by treating intact embryonic cells or cell extracts with proteases, such as trypsin. These experiments showed that the l(2)gl protein was fully protected against proteolysis when the cells were intact but was degraded when the cells were lysed with detergents (D. Strand et al., in preparation). On the basis of these results, we conclude that the l(2)gl protein is bound to the inner surface of the plasma membrane. The exact nature of this binding is not yet known but may result from either posttranslational modification of the l(2)gl protein or interaction with other proteins.

Function of the l(2)gl Protein

The analysis of the amino acid sequence of the putative l(2)gl protein did not provide any direct indication of its function, and several attempts for identifying structural homologies with protein sequences in data bases were equally unsuccessful until we got indications that the l(2)gl protein was associated with the inner face of the plasma membrane and can be metabolically labeled with [^{32}P]phosphate. On this basis we searched for conserved motifs among proteins exhibiting a similar intracellular localization. This comparison revealed the presence of three reiterated motifs in the central region of the l(2)gl protein that show similarities with repeats characterizing β-subunits of G-proteins (*61*) and displaying the consensus sequence:

LxxGHxxx(I/V)xx(I/L/V)xxxxx
(G/S)xx(I/L/V)x(S/T)xDxx(I/L/V)WD

Five such repeats with a 42 to 64 amino acid unit interval are usually present in the β-subunits of G proteins whereas, in l(2)gl, we have only identified three repeats that are reiterated with an 86 to 89 amino acid interval. The function of the repeat structure is unknown, but, due to its presence in protein unrelated to G protein such as the yeast PRP4 protein that is involved in the process of RNA splicing, it has been speculated that the complex motif may play a role in protein-protein interaction (*61*).

In conjunction with the finding that the p127 protein binds to the inner face of the plasma membrane, the presence of these motifs suggests that the *l(2)gl* protein may establish contact with other proteins and through these interactions may play a role in a signal pathway that ultimately controls cell proliferation and cell differentiation.

Spatio-Temporal Expression Patterns of *l(2)gl* and Critical Period for Establishment of Neoplasia

Northern blot analysis has revealed that the *l(2)gl* gene is essentially active during embryogenesis and during the larval-pupal transition phase. Interestingly,

these two phases of *l(2)gl* gene expression correspond to the two major periods of cell proliferation during Drosophila development. It is therefore important to determine the respective contribution of each period of *l(2)gl* expression in relationship with tumorigenicity.

At first, the appearance of tumors that become visible during the extended larval life of the *l(2)gl* mutant animals would suggest that the control of tumorigenesis takes place during the second period of gene expression. Such a hypothesis would also implicate that *l(2)gl* acts as a direct repressor of cell proliferation. However, this hypothesis is contradicted by two observations. First, the *l(2)gl*-deficient embryos are able to complete their embryogenesis normally with no apparent morphological alteration. Second, transplantation of *l(2)gl* embryonic cells into the abdomen of adult hosts has revealed that these cells produce tumors and are therefore committed to neoplastic growth.

In order to determine more precisely the critical phase for the establishment of neoplasia during Drosophila development, we have examined the tumorigenicity of clones of *l(2)gl*-deficient cells that have been induced at different developmental periods. Analysis of *l(2)gl* mosaic animals indicated clearly that neoplastic growth can only take place in clones of cells that have lost the *l(2)gl* gene in preblastoderm syncytical embryos prior to any expression of the *l(2)gl* gene. By contrast, *l(2)gl* clones that are produced at later embryonic stages do not exhibit a neoplastic phenotype but are unable to complete differentiation. Finally, *l(2)gl* clones arising during larval development show a nearly normal or normal differentiation. These data indicate strongly that the critical period for the establishment of *l(2)gl* neoplasia occurs during embryogenesis (*55*).

Analysis of the embryonic expression of *l(2)gl* as revealed by *in situ* hybridization and immunostaining showed that *l(2)gl* is ubiquitously expressed in all cells during early embryogenesis from the cellular blastoderm stage up to the germ band extension stages (about 8.5 hr of embryonic development). During this period, *l(2)gl* expression remains uniform and relatively intense over all embryonic cells (*55,58*). At the time for the dorsal closure (about 110 hr of development), the l(2)gl transcripts become gradually restricted to the epithelial cells of the midgut where they persist until the end of embryogenesis and disappear in all other tissues (*55,58*). Immunostaining analysis revealed that the pattern of l(2)gl protein expression follows generally the pattern of l(2)gl transcription. In early embryos, the l(2)gl protein is evenly distributed in all embryonic cells. In late embryos, the protein is only detected in the midgut cells and in central nervous system. The accumulation of the l(2)gl protein in the axons indicate that the l(2)gl protein turnover and/or translational efficiency of the l(2)gl transcripts in the neuroblasts may be different from that in other tissues.

Thus, *l(2)gl* displays a complex pattern of expression that is not only limited to regions of embryonic map predicted to be the domain of *l(2)gl* activity. *l(2)gl* is first ubiquitously expressed during early embryogenesis and then becomes limited to domains that do not exhibit any apparent pattern of damage in the absence of *l(2)gl*.

With respect to tumorigenesis, it is only during early embryogenesis that a correlation can be established between *l(2)gl* expression and the tumor phenotype. At this time *l(2)gl* is expressed in the epidermal and neural precursors, the progeny of which are tumorigenic in *l(2)gl*-deficient animals. This correlation is further strengthened by the results of the mosaic analysis that showed that the early embryonic expression of *l(2)gl* is necessary for preventing tumorigenesis. Furthermore, this period of gene expression seems to be also important for the other tissues that exhibit nontumorigenic phenotypic alterations.

The information that we have gained from the molecular analysis of the *l(2)gl* gene leaves a number of unresolved questions and paradoxes. Although *l(2)gl* is expressed in a wide variety of tissues, its inactivation affects only a limited number of tissues, whereas most of the tissues are unaffected. Reduced growth capacities are observed in some cell lineages, such as the germ line, the imaginal cells of the salivary glands, and the prothoracic cells of the ring gland, whereas neoplastic growth occurs only in the imaginal disc cells and the neuroblasts and ganglion mother cells of the presumptive adult optic centers in the larval brain.

Equally perplexing is the delayed appearance of the tumors. The malignant pattern of growth only becomes visible during late larval life, although it can be directly correlated with the absence of *l(2)gl* gene expression during early embryogenesis. Also intriguing is the absence of direct visible effect of the lack of *l(2)gl* expression on the embryonic development that appears to proceed normally. In particular, no overgrowth of any tissue can be observed, indicating that the potential tumorigenic cells first follow a normal pattern of development with cessation of cell division during late embryogenesis similar to that in wild-type embryos.

How the *l(2)gl* gene product controls cell growth and cell differentiation clearly depends on the cellular context in which *l(2)gl* is expressed. It is possible to conceive that the l(2)gl protein plays a broad role in linking plasma membrane-bound receptors to intracellular effector proteins. These components may vary depending on the cell type. Therefore, it will be important to determine the nature of these components and to define their contribution in the establishment of tumorigenesis.

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